#96

THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

JAN 31 1974

Date: 1-24-74

1. Principal Investigator (give title and degrees):

Nathan H. Sloane, Professor of Biochemistry, B.S., M.P.H., Ph.D.

Audrey N. Roberts, Professor of Microbiology, B.S., M.A., Ph.D.

2. Institution & address:

The University of Tennessee Medical Units
62 Dunlap Street
Memphis, Tennessee 38163

3. Department(s) where research will be done or collaboration provided:

Department of Biochemistry and Department of Microbiology

4. Short title of study:

Effect of Benzo(α)pyrene and Derivatives on Mammalian Lung Cells

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: July 1, 1977

7. Brief description of specific research aims:

1. Determine whether metabolic hydroxymethylation of benzo(α)pyrene to the 6-hydroxymethyl derivative represents a pathway to form a more proximate carcinogen from the polycyclic hydrocarbon .

2. Determine the biological properties of benzo(α)pyrene carcinogenic polycyclic hydrocarbon derivatives in vitro using primary cultures prepared from human lung tissue, embryonic human lung tissue, rat lung tissues, and mouse lung tissues. These latter studies will utilize dwarf mutation mice (dw) and their normal sized litter mates.

3. Determine the effects of benzo(α)pyrene on primary and continuous cell lines in vitro in the presence and absence of cytochrome P-450 inhibitors using cytotoxicity and cell transformation as quantitative biological parameters.

4. Determine levels of the benzo(α)pyrene metabolizing enzymes in the lungs and the livers of the dwarf mice (which are tumor resistant) to correlate these enzymatic activities with tumor susceptibility.

5. Determine the binding of tritium labeled benzo(α)pyrene, 6-hydroxymethyl-benzo(α)pyrene, 6-methylbenzo(α)pyrene and 3-hydroxybenzo(α)pyrene to DNA, RNA and protein fractions of lung cells grown in culture in the presence and absence of cytochrome P-450 inhibitors.

8. Brief statement of working hypothesis:

Previous studies (NHS)have shown that the mammal possesses 2 metabolic pathways for the formation of aryl hydroxymethyl compounds: 1) oxidation of the aryl side chain methyl group to the hydroxymethyl group; 2) direct hydroxymethylation of the benzene ring - an aryl hydroxymethyl synthetase reaction. The synthetase reaction is independent of cytochrome P-450. However, the oxidation of the aryl side chain methyl group is cytochrome P-450 mediated. Furthermore, the hydroxylation of the benzene ring of polycyclic hydrocarbons also requires the participation of cytochrome P-450. Therefore, it is now possible to study the direct biological effect of the hydroxymethyl synthetase reaction of benzo(α)pyrene in the absence of the hydroxylation reaction. Theoretically the aryl hydroxymethyl compound should be a more proximate carcinogen as postulated by Dipple, Lawley and Brookes (Europ. J. Cancer 4: 493, 1968). Flesher and Sydnor showed that rat liver homogenates hydroxymethylate benzo(α)pyrene to the 6-hydroxymethyl derivative and these investigators also demonstrated that this derivative is carcinogenic. Sloane and Davis (manuscript submitted for publication) showed that the carcinogenic hydrocarbon is indeed hydroxymethylated by the soluble enzyme system obtained from liver microsomes.

9. Details of experimental design and procedures (append extra pages as necessary)

1. Study of the Enzymatic Hydroxymethylation of Benzo(α)pyrene

a. We propose to prepare lung and liver homogenates in order to determine the concentration of the 6-hydroxymethyl synthetase. We shall utilize human tissues (lung and liver) as well as liver and lung of both the normal mouse and the dw litter mate that has been shown to be resistant to chemical carcinogenesis.

Bielschowsky and Bielschowsky (Brit. J. Cancer 13: 302, 1959; Brit. J. Cancer 14: 195, 1960; Brit. J. Cancer 15: 257, 1961) studied the effect of chemical carcinogens in the pituitary dwarf mouse. These investigators demonstrated that these mice were resistant to some carcinogens, namely 2-aminofluorene and dimethylbenzanthracene, whereas no resistance was shown by these mice to the carcinogen, methylcholanthrene. The hypophysectomized rat did show formation of sarcomas induced by benzo(α)-pyrene (Zamurovitch, Onocolgia 6: 190, 1953).

b. The microsomal fraction of the homogenate will be prepared and fortified with the above cofactors, boiled liver juice, and/or folate derivatives as described below.

c. The soluble microsomal enzyme fraction of these microsomes will be prepared by the method of Sloane and Heinemann (Biochim. Biophys. Acta 201: 384, 1970). We have previously shown that hydroxymethylation of the benzene ring is accomplished by an enzyme system that is present in this soluble microsomal fraction; the aryl hydroxymethyl synthetase requires a reduced pyridine nucleotide and a macromolecule that donates the C-1 fragment.

d. We shall study the possible role of folate derivatives on the hydroxymethylation of benzo(α)pyrene by the enzyme system. We shall investigate the effect of the addition of N^{10} formyl tetrahydrofolate (Robinwitz and Priger, J. Biol. Chem. 237: 2898, 1962), N^{5} formyl tetrahydrofolate, N^{5} - N^{10} methylene tetrahydrofolate (the coenzyme of serine hydroxymethylase, thymidylate synthetase, and deoxycytidylate hydroxymethylase) to the enzyme system. The coenzyme, N^{5} - N^{10} methylene tetrahydrofolate will be prepared by the method described by Hunnekens,

Ho, and Scrimgeous in Methods in Enzymology, Vol. VI, pp. 807-812, Academic Press, 1963).

e. The enzyme will then be purified utilizing the conventional techniques of enzyme purification. These include separation of nucleic acids, ammonium sulfate fractionation, acetone and/or alcohol fractionation, ion exchange cellulose chromatography, Sephadex chromatography (G-25 to G-200) and/or Bio Gels to fractionate proteins according to molecular weights and DEAE-Sephadex anion exchange chromatography, and preparative gel electrophoresis. Evidence for the successful use of these conventional techniques of enzyme purification is provided in Publications of N. H. Sloane; 8,9,12,13,19,20,21,27 and 28.

f. The hydroxymethylation of benzo(α)pyrene by the enzyme system will be determined by the isolation of the labeled product 6-hydroxymethylbenzo (α)pyrene by the use of thin layer chromatography as described by Flesher and Sydnor (63rd Annual Meeting of the Am. Assoc. for Cancer Research) utilizing the precursor compound that had been tritium labeled by catalytic exchange. The isolated labeled 6-hydroxymethylbenzo(α)-pyrene will be counted so that we shall have a quantitative assay for this hydroxymethylation. This type of assay was successfully utilized in our previous studies on the hydroxymethylation of benzene.

Benzo(α)pyrene is commercially available, whereas the 6-hydroxy-methylbenzo(α)pyrene has been prepared by the procedures of Flesher and Sydnor. It is not probable that methylation of benzo(α)pyrene precedes the formation of the hydroxymethyl derivative, because Sloane and Heinemann showed that S-adenosyl-L-methionine was not involved in the hydroxymethylation of benzene to benzyl alcohol.

We shall attempt to correlate the concentration of benzo(α)pyrene hydroxymethyl synthetase in the lung and liver of carcinogen susceptible and resistant dw mice as well as determine the concentration of the enzyme in normal human lung and neoplastic lung tissues. Dr. Roy Page of the Page Clinic (Memphis, Tennessee) will provide human tissues for these studies.

2. Study of the Enzymatic Hydroxylation of Benzo(α)pyrene

a. We propose to determine the concentration of the benzo(α)pyrene hydroxylases (specifically 3-hydroxybenzo(α)pyrene in the tissue systems discussed above under the Section A. 1.a. These studies will be performed to correlate the concentration of the hydroxylase in the lung and liver of carcinogenic susceptible and resistant dw mice.

b. The concentration of the hydroxylase will be determined by the method of Conney et al. (A. H. Conney, E. C. Miller and J. A. Miller, J. Biol. Chem. 228: 753, 1957).

c. Since the hydroxylation of benzo(α)pyrene requires cytochrome P-450, we shall study the role of both mitochondrial and microsomal cytochrome P-450 on the oxidation reaction. During the summer months of 1969, 1970 and 1973, N. H. Sloane worked in the Laboratory of Population Sciences, Harvard University School of Public Health with Dr. H. A. Salhanick, on the isolation of cytochrome P-450 from Pseudomonas

putida. We showed that P-450 CAM will not act as the steroid hydroxylating enzyme in beef adrenal mitochondria, in which the adrenal P-450 was destroyed by sonication. Furthermore, we showed that inhibitors of Ps. P-450 CAM (as evidenced by spectral changes) also inhibited the P-450 of bovine corpus luteum, thus indicating that specificity does not exist in the inhibitor binding sites.

d. We have recently obtained evidence to indicate that the enzymatic activation of oxygen for the side chain hydroxylation of the aryl methyl group to the aryl hydroxymethyl group is mediated by a mixed function oxidase; the reaction studied was the conversion of toluene to benzyl alcohol. The hydroxylation reaction requires molecular oxygen NADPH and cytochrome P-450. Evidence for the role of cytochrome P-450 for this oxidation was obtained using diverse cytochrome P-450 inhibitors. The compounds studied were carbon monoxide, metyrapone, 1-benzylimidazole and aminoglutethimide. These experiments employed the sonicate of rat liver microsomes prepared from 50 to 60 day old female Holtman rats.

e. The cell cultures to be studied will allow comparisons of carcinogen effects on normal and malignant human lung cells as well as on lung cells derived from normal and dwarf mice.

B. In Vitro Cell Culture Studies

1. Carcinogen Effects on Lung Cell Cultures

a. The effects of benzo(α)pyrene, 3-hydroxybenzo(α)pyrene, k region epoxide of benzo(α)pyrene, 6-hydroxymethylbenzo(α)pyrene and 6-methylbenzo(α)-pyrene will be determined in cell cultures initiated from normal and malignant human lung tissues, human embryonic lung and lung tissue from normal and dwarf mice. The cell culture techniques are described below. The effects of these compounds will be determined in the presence and absence of cytochrome P-450 inhibitors in order to delineate the role of the aryl hydroxymethyl synthetase reaction on the chemical carcinogenesis. The effects of the cytochrome P-450 inhibitors on the hydroxylation of benzo(α)pyrene will be determined by the method of Conney et al. (J. Biol. Chem. 228: 753, 1957).

b. The effects of these inhibitors on the aryl side chain hydroxylation of 6-methylbenzo(α)pyrene to the 6-hydroxymethyl derivative will be determined by the method of Sloane and Davis (manuscript submitted for publication).

c. Collection of normal and malignant human lung and human embryonic tissues. All human tissues will be provided through the courtesy of Roy C. Page, M.D. of the Page Clinic, Memphis, Tennessee. All normal and malignant adult tissue will be histologically examined by Surgical Pathologists as a part of routine surgical procedure to confirm normalcy and malignancy, respectively. Embryonic tissues will be obtained from first and second trimester human fetuses.

d. Cell culture techniques. The human tissue specimens will be collected in Hanks' balanced salt solution (BSS) containing penicillin (50 units per ml), streptomycin (50 µg per ml) and mycostatin (40 units per ml) and transported to the laboratory on ice. All human and mouse tissues will be minced and washed with Hanks' BSS. Primary cultures will be initiated from tissue minces in 250 ml milk dilution bottles, planting 25 to 30 tissue fragments approximately two mm^2 per bottle. All cells will be cultured in Eagle's MEM containing 10 per cent fetal calf serum and antibiotics at 37 C under 5 per cent CO_2. When complete monolayers are obtained, the cells will be subcultured by treatment with EDTA alone or in combination with 0.025 per cent trypsin.

Carcinogen studies will be performed in 60 x 15 mm Falcon plastic petri plates seeded with approximately 1×10^6 cells per plate. Cover glass cultures to be used for autoradiography or routine morphological studies also will be grown as monolayers in petri plates.

e. Cytochrome P-450 Inhibitors. Preliminary studies will determine the effects of cytochrome P-450 inhibitors on the plating efficiency and growth of each cell line. The inhibitors, aminoglutethimide, metyrapone, 4-phenylimidazole, 1-benzylimidazole and SKF 525A, will be tested at final concentrations of 1 to 2×10^{-4} M. For tests on plating efficiency, cells will be seeded in 5.0 ml of medium containing inhibitor and incubated for 24 hours at 37C under 5 per cent CO_2. The medium then will be withdrawn and the monolayers washed 2X with 5.0 ml of Hanks' BSS to remove unbound cells. The cells will be detached from the plastic surface with trypsin and counted in a hemacytometer. The number of cells present will be compared with the number from untreated cultures, both conducted in triplicate.

To study the influence of inhibitors on cell growth, the cells will be seeded in culture plates both in the presence and absence of inhibitor. Cultures seeded in the absence of inhibitor will be incubated for approximately 24-48 hours, or until the monolayers are approximately 50 per cent confluent, at which time inhibitors will be added. The cell yields from each test system will be determined in triplicate at 24 hour intervals through 6 days of incubation and compared with the cell yields from untreated control cultures. Only inhibitors that have no effects on plating efficiency and cell growth will be used in all subsequent carcinogen studies.

Preliminary studies in our laboratory have shown that metyrapone, 4-phenylimidazole and 1-benzylimidazole have no adverse effects on the plating efficiency and growth of human embryonic lung cells and epidermoid lung carcinoma cells at concentrations of 1 and 2×10^{-4} M.

f. Carcinogen effects on in vitro cell cultures

1) Plating efficiency and growth rate. The effects of carcinogens on the plating efficiency and growth rate of each cell line will be studied in the presence and absence of cytochrome P-450 inhibitors.

Details of methodology are described above. Since the carcinogens will be prepared in dimethylsulfoxide (DMSO), both untreated cultures and cultures treated with an equivalent concentration of DMSO will serve as controls. These studies also will include microscopic examination of cover glass cultures after staining with methyl green pyronine and may Grunwald-giemsa for observation of possible variation in cell morphology.

2) Localization of tritium labeled carcinogens. In addition to analyses of the tritium concentrations in isolated subcellular fractions (described below), the cellular patterns of tritium localization will be observed in autoradiographs of cover glass cultures. Autoradiographs will be prepared using Kodak NTB3 liquid emulsion, exposed, developed and stained as described by Roberts (Am. J. Pathol. 49: 889, 1966).

3) Effects of carcinogens on DNA synthesis in vitro. The influence of the carcinogens on DNA synthesis and generation times in the presence and absence of cytochrome P-450 inhibitors will be determined in each cell line by studies on the incorporation of tritiated thymidine (TdR^3H). Using unlabeled carcinogens, the cultures will be pulsed for 4 hours with 0.5 µCi per ml of TdR^3H at 24 hour intervals through 6 days of incubation. The culture processing, DNA and protein extraction and scintillation counting to measure uptake of TdR^3H will be performed as described by Capary and Hughes (J. Immunol. Methods 1: 263, 1972). All analyses will be performed in triplicate cultures as described above. Untreated cultures and DMSO treated cultures will serve as controls.

At each study interval, cover glass cultures will be prepared for autoradiographic determination of the per cent of labeled cells. The number of labeled cells per culture thus will be compared and correlated with the quantitative assays of TdR^3H incorporation by the total cell population.

The generation time will be measured on the biphasic curve representing the percentage labeled metaphases obtained after the pulse labeling of actively growing cells, as described by Quastler and Sherman (Exp. Cell Res. 17: 420, 1959). The periods of exponential growth will be established with untreated control cultures of each cell line to be investigated.

4) Transformation of in vitro cultures. The potential for induction of malignant transformation by the carcinogens in cultures of human embryonic lung and of normal and dwarf mouse lung will be monitered. These studies will require the maintenance of carcinogen-treated cells in continuous cultures for a period of several weeks. Periodic microscopic examination of the cultures will reveal the commonly used criteria for transformation, such as a change in morphology or a change in the social behavior of the transformed cells. These changes include palisading of cells with loss of contact inhibition. These converted cells often form high density colonies which can be seen macroscopically. Colony counts will be performed to obtain a quantitative measure of transformation (Hartley and Rowe, Proc. Nat. Acad. Sci. 55: 780, 1966).

The transformed colonies will be isolated by microcloning techniques, as described by Robb (Science, 170: 857, 1970). Cell cultures derived from the cloned colonies will be characterized by studies on growth rate and generation time and by karyology (in Tissue Culture Methods and Applications, edited by P. F. Kruse and M. K. Patterson, Jr., Chapter 15, 1973).

Cell cultures derived from transformed clones will be further characterized for chemical oncogensis by tests for tumor induction in the proper host. Successful systems reported include the hamster embryo cell system (Berwald and Sachs, J. Nat. Cancer. Inst. 35: 641, 1965), the C^3H mouse prostate system (Chen and Heidelberger, Intern. J. Cancer 4: 166, 1969) and 3T3-like cell lines derived from C3H mouse embryo cells (DiPaolo, Takano and Popescu, Cancer Res. 32: 2686, 1972). In these systems, the transformed clones give rise to fibrosarcomas on inoculation into the proper host.

The inbred normal and dwarf mouse systems proposed in our studies will offer suitable models in which to test the carcinogen transformed mouse clones for tumorigenesis, thus confirming the malignant properties of the transformed cells.

The growth of human malignant tumor tissue in irradiated, thymectomized mice also has been reported (Castro, Nature New Biol., 239: 83, 1972). In addition, cultured human malignant cell lines have been shown to form progressively growing tumors in thymectomized rats simultaneously treated with antirat lymphocyte serum (Plata et al., J. Natl. Cancer Inst., 50: 849, 1973). We propose, therefore, to test transformed cloned cells from carcinogen-treated human embryonic lung cell lines for tumor induction in immunosuppressed mice.

2. DNA Labeling by Metabolism of Tritiated Carcinogens in Lung Cells Grown in Culture.

a. We shall determine the amount of tritiated compound bound to the DNA fractions of lung cells grown in culture in the presence and absence of cytochrome P-450 inhibitors in order to study the role of cytochrome P-450 mediated hydroxylations and cytochrome P-450 independent hydroxymethylation on the activation of carcinogenic polycyclic hydrocarbons delineated above. The tritiated hydrocarbons will be prepared from the parent hydrocarbons by the Wilzbach tritium exchange technique and/or catalytic exchange labeling to be performed by INC Pharmaceuticals, Inc., 2727 Campus Drive, Irvine, California 92664.

The techniques to be employed in these experiments are those described by Rayman and Dipple (Biochemistry 12: 1538, 1973).

One of us (Nathan Sloane) will be at the Jackson Laboratories, Bar Harbor, Maine during June, July and August, 1974, to study the isolation of DNA fractions by density gradient centrifugation and also to work with the effect of ACTH on the liver RNA/DNA ratio of dwarf mice compared to the congenic litter mates. Dr. Sloane will be a Visiting Investigator sponsored by Dr. Harry Chen.

3. RNA and Protein Labeling by Metabolism of Tritiated Carcinogens in Lung Cells Grown in Culture

We shall determine the amount of tritiated compound bound to the RNA and protein fractions of cells grown in culture in the presence and absence of cytochrome P-450 inhibitors. The techniques to be employed in these studies will be those described by Duncan and Brookes (Int. J. Cancer 6: 496, 1970).

C. Effects of Benzo(α)pyrene Polycyclic Hydrocarbons and Derivatives on Tissue Culture Cells: A Brief Literature Review.

Recent evidence suggests strongly that the epoxide of polycyclic hydrocarbons, which is formed by a mixed function oxidase in microsomes, is a proximate carcinogen (Sims, Biochem. J. 84: 558, 1962; Boyland and Sins, Biochem. J. 95: 778, 1965; Silkirk, Huberman and Heidelberger, Biochem. Biophys. Res. Commun. 43: 1010, 1971).

Gelboin et al. (Proc. Nat. Acad. Sci. U.S. 64: 1188, 1969) showed a direct correlation between the level of the enzyme benzo(α)pyrene hydroxylase and the susceptibility of the tissue culture cells to cytotoxicity produced after treatment with benzo(α)pyrene; furthermore these investigators showed that 3-hydroxybenzo(α)pyrene was cytotoxic to cells that were resistant to the cytotoxic effect of benzo(α)pyrene per se.

However, Marquardt et al. (Proc. Am. Assoc. for Cancer Res. Abs. 27, 1973) reported that the k region epoxides of 7, 12-dimethylbenz(α)anthracene and 7-hydroxymethylbenz(α)anthracene were more lethal but produced fewer transformed foci than the parent hydrocarbon with mouse prostate fibroblasts. Thus the involvement of epoxides in carcinogenesis by these hydrocarbons remains in doubt.

These latter results appear contradictory to data reported by Gelboin and Wiebel (Science 170: 169, 1970); who showed that 9, 10-dimethylbenzanthracene tumorigenesis was inhibited by 7, 8-benzoflavone, an inhibitor of aryl hydrocarbon hydroxylase. However, Wheatley (Brit. J. Cancer 2: 787, 1968) showed that SKF-525-A (an inhibitor of cytochrome P-450) enhanced mammary tumorigenesis by 7, 12-dimethylbenz(α)anthrancene. However, metabolism of certain methyl derivatives involves formation of hydroxymethyl derivatives which may also be proximate carcinogens (Boyland, Sims, Huggins, Nature, 207: 816, 1965; Flesher, Sydnor, Cancer Res. 31: 1951, 1971). Epoxidation of methyl polycyclic hydrocarbons appears minimal (Boyland and Sims, Biochem. J. 95: 780, 1965).

The contradictions implicit in these two mechanisms of carcinogenesis by hydrocarbons have been mitigated by a recent finding of Flesher and Sydnor (Abs. p. 55, No. 217: 63rd Annual Meeting Proc. Am. Assoc. for Cancer Research, May 4-6, 1972, Boston, Massachusetts) who demonstrated the 6-hydroxymethyl derivative as one of the metabolites of benzo(α)pyrene. Thus hydroxymethylation may be the route to form a proximate carcinogen, which is common to polycyclic hydrocarbons. This metabolic pathway should be investigated further.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The University of Tennessee, Department of Biochemistry, School of Medicine has office, laboratory, library, shop, stockroom and coldroom facilities to carry out the proposed research. The following items are available: Amino acid analyzer, chromatographic equipment, high voltage paper electrophoresis equipment, animal facilities, UV and IR spectrometers, Model E ultracentrifuge, liquid scintillation counting equipment and Craig automatic counter-current distribution apparatus.

Dr. Roberts' research facilities in the Department of Microbiology consist of 265 sq. ft. of office space and 1130 sq. ft. of laboratory space. Adquate facilities are available for autoradiography, fluorescence microscopy, tissue culture, glassware preparation and the housing and care of laboratory animals. The following equipment is also available: Sorvall RC2-B superspeed refrigerated centrifuge with 3 rotors, Isco Fraction Collector, DG fractionator, UV monitor and recorders equipped with flow cells, DB and column fractionation apparatus, Bausch and Lomb refractometer, Nuclear Chicago Scintillation Spectrometer, Gilford Model 240 spectrophotometer, Zeiss bright field and fluorescent microscope, AO bright field microscope, immunoelectrophoresis apparatus, Sorvall GLC-1 centrifuge with 2 rotors, low speed counter-top centrifuge, Mettler microbalance and small laboratory balances. Torsion balance and pH meter, microtissue homogenizer, -20C and -70C freezers, 5C refrigerators, 37 C incubators, waterbaths, Virtis freeze-dry apparatus, Spinco Model L preparative ultracentrifuge, Packard Tri-Carb liquid scintillation spectrometer, Spinco L2B-65 preparative ultracentrifuge and several rotors, preparative gel electrophoresis apparatus, Sonifier, liquid nitrogen cell culture storage tank, and two large CO_2 incubators.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Nathan Sloane, Ph.D.	40	0
Audrey Roberts, Ph.D.	30	0
Technical		
Helen Hill, Laboratory assistant (Salary $5,200; fringe @ 8.5% $442)	100	5,642
Tissue Culture technician, B.S. (to be recruited) (Salary $7,200; fringe @ 8.5% $612)	100	7,812
Chemical research assistant (to be recruited) (Salary $5,400; fringe @ 8.5% $459)	100	5,859
	Sub-Total for A	$ 19,313

B. Consumable supplies (by major categories)	
Tissue culture glassware and plastic disposables	2,400
Tissue culture media and serum (One liter of 10X medium and one liter fetal calf serum per wk)	5,000
Biochemical supplies	2,000
Vivarium charges for animal care and animal costs	1,000
Sub-Total for B	10,400

C. Other expenses (itemize)	
Travel (to attend scientific meetings)	
Sub-Total for C	800
Running Total of A + B + C	30,513

D. Permanent equipment (itemize)

None

Sub-Total for D	0
E	4,577
Total request	35,090

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	20,283	10,500	800	0	4,738	36,321
Year 3	21,297	10,500	800	0	4,890	37,487

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Ring Hydroxymethylation in Hydrocarbon Carcinogenesis	American Cancer Society Grant BC 137	$26,000	July 1, 1973- June 30, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator s Nathan H. Sloane, Ph.D.
Audrey N. Roberts, Ph.D.

Typed Name _____

Signature Nathan H. Sloane Audrey N. Roberts Date 1/24/74

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